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A novel missense mutation p.S305R of *EDA* gene causes XLHED in a Chinese family

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ABSTRACT

X-linked hypohidrotic ectodermal dysplasia (XLHED) can be characterized by hypohidrosis, sparse hair, hypodontia, and characteristic facial features and is usually caused by mutations of ectodysplasin A (*EDA*) gene located on the X chromosome. In this study, we examined a HED pedigree and studied the molecular genetics of the disease. A novel missense mutation was revealed by direct sequencing analysis in the *EDA* exon 7 (c.913 A > C, p.S305R). The impact of the mutation on the protein was studied in vitro in human embryonic kidney 293 T cells transfected with mutant or wild type forms of *EDA*. The mutant-type EDA1 protein showed impaired solubility comparing with wild-type EDA1. This novel missense *EDA* mutation was considered to be the cause of HED in the pedigree reported here. Our findings, combined with those reported elsewhere, provide an improved understanding of the pathogenic mechanism of HED as well as important information for a genetic diagnosis.

1. Introduction

Ectodermal dysplasia (ED) contains a group of more than 170 types of congenital disorders resulting from abnormal development of ectoderm-derived structures (Pinheiro & Freire-Maia, 1994; Priolo & Lagana, 2001). The most common type of ED, hypohidrotic ectodermal dysplasia (HED), occurs in 1 in 10,000–100,000 births (Chassaing, Bourthoumieu, Cossee, Calvas, & Vincent, 2006; Itin & Fistarol, 2004). HED is characterized by hypohidrosis, hypotrichosis, hypodontia, and characteristic facial features. The common clinical signs include dry skin, sparse hair, deformed teeth, diminished or absent of eccrine function and characteristic facial features including frontal bossing, chin prominence, saddle nose, maxillary hypoplasia, low-set ears, wrinkles, and periorbital hyperpigmentation. Three inheritance patterns of HED are known: X-linked recessive, autosomal recessive, and autosomal dominant, corresponding to the main causative genes (*EDA*, *EDAR* and *EDARADD*). The X-linked HED (XLHED) is caused by mutations of *ectodysplasin A (EDA)* gene (Genbank accession number: NM 001399) located within the X q12-q13.1 region, causing an X-linked recessive pattern of inheritance (Deshmukh & Prashanth, 2012; Kere et al., 1996). The hemizygous males are often most severely affected, showing the “classic” clinical features, while heterozygous females generally show normal or moderately affected features (Chassaing et al., 2006; Clarke, Phillips, Brown, & Harper, 1987; Deshmukh & Prashanth, 2012; Itin & Fistarol, 2004; Kere et al., 1996; Savasta, Carlone, & Castagnoli, 2017).

The EDA-A1 protein which contains 391 amino acids, is the most common and also the largest product of the *EDA* gene (Podzus, Kowalczyk-Quintas, Schuepbach-Mallepell, Willen, & Staehlin, 2017). It is a trimeric type II transmembrane protein composed of a transmembrane domain, a furin protease recognition site, a 19 repeat Gly-X-Y collagenous domain as well as a tumor necrosis factor (TNF) homology C-terminal domain (Monreal, Zonana, & Ferguson, 1998).

To date, more than 300 mutations of the *EDA* gene have been reported (Cluzeau et al., 2011; Trzeciak & Koczorowski, 2016), including missense, nonsense and splicing mutations as well as small deletions (Wohlfart, Hammersen, & Schneider, 2016; Yin et al., 2013). 60–70% of the mutants have been found in exons 7,8 and 9 which encode the TNF homology subdomain. 25 of the 139 missense mutations reported in the database of single nucleotide polymorphisms (SNP) of the National Center for Biotechnology Information (NCBI) are pathogenic.

In this study, we describe the clinical features and molecular characterization of a novel missense mutation identified in a Chinese family with XLHED. We also performed in vitro functional experiments to

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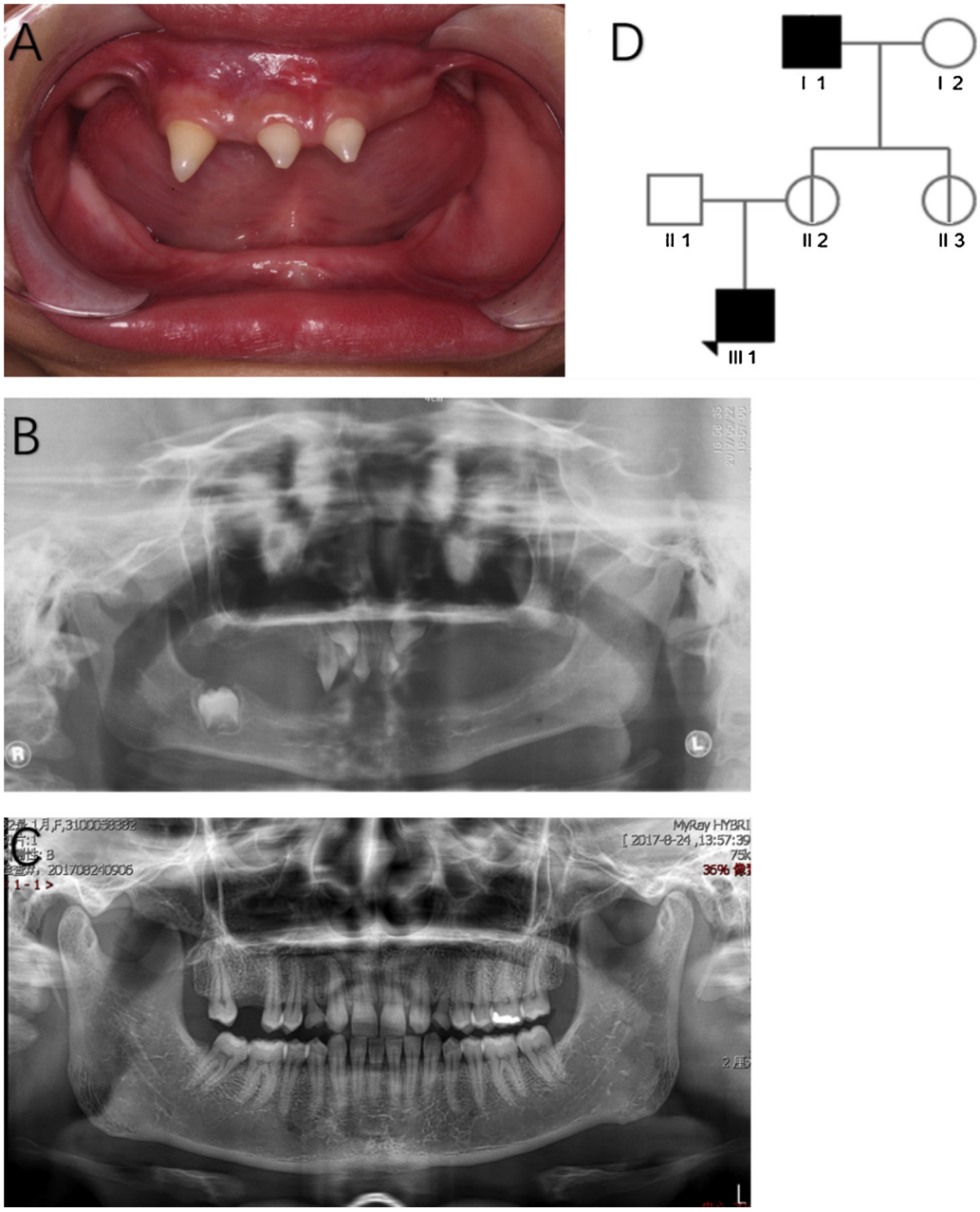

Fig. 1. Phenotypic features of members of the pedigree. A and B: Intraoral photograph and panoramic radiograph of the proband showing the reduced number of deciduous and permanent teeth. C: Panoramic radiograph of the mother of the proband showing the absence of permanent upper canines. D: Pedigree of the family with an X-linked recessive inheritance pattern. Affected individuals are represented by filled squares (males), while non-penetrant carriers are represented by circles with a vertical line (females).

elucidate the molecular mechanisms underlying the effects of this mutation.

2. Materials and methods

2.1. Subjects

The proband were referred to the School and Hospital of Stomatology, Peking University (China) for oligodontia. After examination by pediatric dentistry specialists, the proband were diagnosed as XLHED. Clinical images and panoramic radiographs of jaws were obtained by standard procedures. The guardian provided informed consent to the genetic studies. This study has been approved by Peking University school of stomatology institutional review board (approval number: PKUSSIRB-201628059).

2.2. Mutation analysis

Blood samples were obtained from the proband and his family members. Genomic DNA was extracted from peripheral blood leukocytes using TIANamp Blood DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. DNA sequences were amplified by polymerase chain reaction (PCR) using specific primers covering the exons and intron–exon boundaries of the *EDA* gene as described previously (Bayés et al., 1998; Fan et al., 2008; Kere et al., 1996). Sanger sequences of individual genes were analyzed using the Chromas chromatogram viewer (http://technelysium.com.au/wp/chromas/) and the National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi). The detected variant was assessed with the mutation prediction tool Mutation Taster (http://www.mutationtaster.

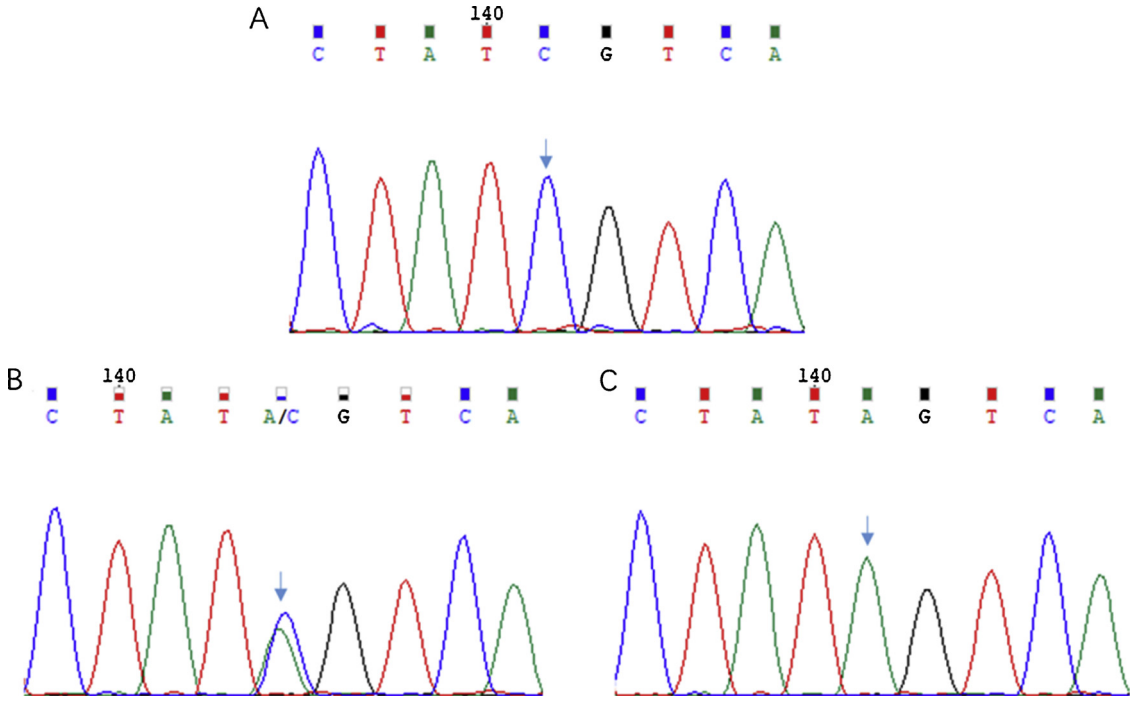

Fig. 2. A: Sequencing chromatogram of the missense mutation c.913 A > C in the *EDA* gene of the proband. B: Sequencing chromatogram of the heterozygous mutation c.913 A > C in the *EDA* gene of the proband's mother. C: Sequencing chromatogram of the *EDA* gene of the proband's father.

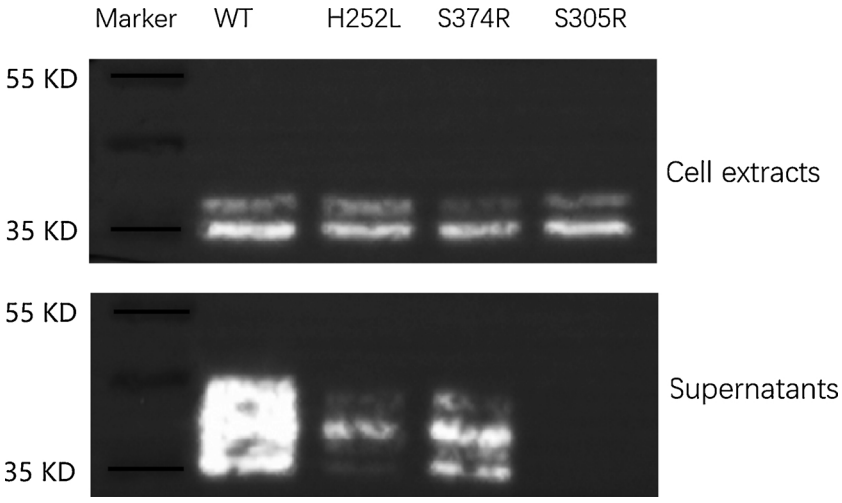

Fig. 3. Expression of wild type and mutated forms of EDA1. Cell extracts and supernatants of HEK 293 T cells transfected with the indicated mutants of FLAG-EDA1 were analyzed by Western blot and revealed with an anti-FLAG antibody. The bands of all four forms showed almost the same brightness in the cell extracts revealing the normal ability of producing intracellular proteins. The weaker bands of H252L and S374R mutants in the supernatants reveal the partially impaired solubility of the protein productions, while the lack of S305R band shows the failure of detecting the soluble protein in the supernatant.

org).

2.3. Construction of EDA expression vectors

Mammalian expression vectors for secreted FLAG-tagged forms of wild-type EDA1 and EDA1 with HED-causing mutation S305R were constructed. EDA1 with HED-causing mutation H252L and non-syndromic tooth agenesis-causing mutations S374R (Schneider et al., 2001), were also constructed to make a contrast. The expression vector sequence contains an HA signal peptide, a FLAG tag, a linker (GGACC CGGACAGGTGCAGCTGCAGGTCGACGAAAATCAG) and the coding sequence for the amino acids 160–391 of EDA1 (the amino acid sequence is as follows: SKSNEGADGPVKNKKKGKKAGPPGPNGPPGPPGPPGPQG-PPGIPGIPGIPGTTVMGPPGPPGPPGPQGPPGLQGPSGAADKAGTRENQ-PAVVHLQGQGSAIQVKNDLSGGVLNDWSRITMNPKVFKLHPRSGELEVL-VDGTYFIYSQVEVYYINFTDFASYEVVVDEKPFLQCTRSIETGKTNYNTCY-TAGVCLLKARQKIAVKMVHADISINMSKHTTFFGAIRLGEAPAS). Construction of above expression vectors was performed by GENEWIZ, Suzhou, China. All constructs were verified by direct sequencing.

2.4. Expression of soluble EDA in cells and supernatants

Vectors encoding soluble, wild-type or mutant FLAG-tagged EDA1 protein were transfected into human embryonic kidney 293 T cells, and the cells were maintained in serum-free OptiMEM(Invitrogen Corporation, Carlsbad, CA, USA) culture medium for 48 h. Subsequently, the cells and supernatants were collected separately. The detection of FLAG-tagged EDA1 proteins in cell lysates and supernatants was then performed by anti-FLAG western blot. The results of Western blots were revealed with adequate horse radish peroxidase-coupled secondary antibodies and ECL.

3. Results

3.1. Phenotypic findings

The proband was a 6-year-old boy who presented with absence of several teeth. Physical examination showed apparently dry skin with hypohidrosis, sparse hair and eyebrows, but normal nails. Other facial features including frontal bossing, chin prominence, maxillary hypoplasia and protuberant lips were also obvious in this patient. Intraoral examination revealed only three cone-shaped upper anterior teeth, a lack of alveolar bone development and ordinary salivary flow. Radiographic examinations demonstrated permanent buds of one mandibular molar and two maxillary central incisors. Moreover, the lack of other tooth buds was observed. Moreover, his maternal grandfather had hypodontia and sparse hair and his mother had hypodontia. The pedigree of the proband and his family members are shown in Fig. 1.

3.2. Mutation analysis results

Direct sequencing revealed the presence of a previously unidentified mutation in the exon 7(c.913A > C, p.S305R) in the proband. The mother of the proband carried the same mutation. This mutation was not found in the healthy members of the family (Fig. 2).

3.3. Expression and solubility assay results

The selected XLHED-causing mutation H252L and hypodontia-causing mutation S374R affecting the TNF homology domain could be expressed as soluble, FLAG-tagged secreted proteins, but mutant S305R protein was entirely retained inside the cells (Fig. 3).

4. Discussion

The transmembrane protein ectodysplasin A is expressed by keratinocytes, hair follicles, and sweat glands. The TNF-related ectodysplasin A pathway plays an important role in embryonic development and ectodermal structure formation (Mikkola & Thesleff, 2003). As a TNF-related ligand, it has been confirmed that *EDA* mutations are responsible for X-linked HED. The human *EDA* gene contains 12 exons, with five different transcripts of the EDA protein produced by alternative splicing according to the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). Among them, the EDA1 transcript, which contains eight exons, is the longest and most abundant product generated by transcription of the *EDA* gene.

Here, we identified a pedigree with HED carrying a novel mutation in the exon 7 c.913A > C in *EDA* gene. It leads to the Ser^{305} of EDA1 protein changing to Arg. There have been over 30 mutations reported in the TNF domain homology subdomain of the EDA1 protein which is necessary and sufficient for receptor binding (Cluzeau et al., 2011; Paakkonen et al., 2001; Schneider et al., 2001). These mutations can affect the receptor binding function of the EDA1 protein by impairing its solubility, quaternary structure or can directly affect binding to the receptor (Schneider et al., 2001). The mutation S305R resulted in insoluble EDA1 that was entirely retained inside the cells, suggesting that it experienced folding or solubility problems. In the Western blot result, FLAG-EDA1 migrates as a doublet. The upper band is N-glycosylated EDA1, whereas the lower band is unglycosylated EDA1 (Schneider et al., 2001), indicating that mutant EDA1 S305R went into the endoplasmic reticulum where it was N-glycosylated. This with the fact that FLAG-EDA1 S305R is not secreted points to a solubility problem in the endoplasmic reticulum, probably consecutive to misfolding. The affected amino acid is probably crucial for proper folding of the monomer, as it occurs in the middle of beta-sheet C, the most conserved among TNF family ligands (Bodmer, Schneider, & Tschopp, 2002), and points inside in the monomer structure (Hymowitz, Compaan, & Yan, 2003). Interestingly, similar to serine 305, mutation of the neighbor residue tyrosine 304 (Y304C), which is also located in the middle of beta-sheet C, also produced an insoluble EDA1 protein. In utero treatment of twins with the Y304C mutation using recombinant EDA1 corrected many features of XLHED (Schneider, Faschingbauer, & Schuepbach-Mallepell, 2018).In conclusion, we identified a novel mutation in the *EDA* exon (c.913A > C p.S305R), and demonstrated its role in resulting in insoluble protein. Thus, we hypothesize that this mutation is the cause of HED in the pedigree described here.

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Declaration of Competing Interest

We have no conflicts of interest to declare.

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